Individual larvae of the zebrafish mutant belladonna display multiple infantile nystagmus-like waveforms that are influenced by viewing conditions

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Abstract: PURPOSE: Infantile nystagmus syndrome (INS) is characterized by involuntary eye oscillations that can assume different waveforms. Previous attempts to uncover reasons for the presence of several nystagmus waveforms have not led to a general consensus in the community. Recently, we characterized the belladonna (bel) zebrafish mutant strain, in which INS-like ocular motor abnormalities are caused by misprojection of a variable fraction of optic nerve fibers. Here we studied intrinsic and extrinsic factors influencing the occurrence of different waveforms in bel larvae. METHODS: Eye movements of bel larvae were recorded in the presence of a stationary grating pattern. Waveforms of spontaneous oscillations were grouped in three categories: "pendular," "unidirectional jerk," and "bidirectional jerk," and the occurrences of each category were compared within and between individual larvae. Moreover, the effects of the characteristics of a preceding optokinetic response (OKR), of the field of view, and of the eye orbital position were analyzed. RESULTS: The different waveform categories co-occurred in most individuals. We found waveforms being influenced by the preceding OKR and by the field of view. Moreover, we found different kinds of relationships between orbital position and initiation of a specific waveform, including pendular nystagmus in a more eccentric orbital position, and differences among jerk oscillations regarding the beating direction of the first saccade or waveform amplitude. CONCLUSIONS: Our data suggest that waveform categories in bel larvae do not reflect the severity of the morphological phenotype but rather are influenced by viewing conditions.

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Title

Nystagmus Waveforms in Zebrafish with Infantile Nystagmus Syndrome Mostly Co-occur in the Same Individual and Are Influenced by Viewing Conditions

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Abstract

Purpose: Infantile nystagmus syndrome (INS) is characterized by involuntary eye oscillations of different waveforms among subjects. Previous attempts to uncover the etiology of nystagmus waveforms have not lead to a general consensus in the community. Recently, we characterized the zebrafish mutant *belladonna* (*bel*), in which INS-like ocular motor abnormalities are caused by misprojection of a variable fraction of optic nerve fibers. Here, we study the intrinsic and extrinsic factors influencing the incidence of different waveforms in *bel*.

Methods: Eye movements of *bel* were recorded in the presence of a stationary grating pattern. Waveforms of oscillations were grouped in three categories: “pendular”, “unidirectional jerk”, and “bidirectional jerk” and the incidence of each category was compared among individual larvae. Moreover, the effects of the characteristics of a preceding optokinetic response (OKR), of the field of view and of the eye orbital position were investigated.

Results: All waveform categories co-occurred in most individuals (16 out of 22 larvae). We found waveforms being influenced by the characteristics of a preceding OKR and by the field of view. Moreover, we found a significant correlation between orbital position and initiation of a specific waveform in a subset of individuals (7 out of 13 larvae).

Conclusions: Our data suggest that waveform categories in *bel* larvae do not reflect the fraction of misprojecting retinal axons but rather are influenced by viewing conditions. A similar association between viewing conditions and waveform might

hold true in human subjects with INS, making it difficult to compare different studies in which stimulus conditions are not identical.

Introduction

Infantile nystagmus syndrome (INS), present at birth or shortly after, is a congenital ocular motor disorder characterized by involuntary conjugate, predominantly horizontal oscillations of the eyes[1-2] which can have a severe effect on occupational and social functioning [3-4]. The prevalence is approximately 2 per 1000 individuals [5]. Eye oscillations can display pendular or jerk waveforms. Pendular nystagmus is a sinusoidal oscillation, jerk nystagmus is characterized by accelerating slow drifts and fast resetting phases (saccades). Although INS can be idiopathic, it is often associated with visuo-sensory abnormalities such as fovea hypoplasia, misprojecting optic nerve fibers, and aniridia[6]. Attempts to cluster INS according to the underlying condition and the oscillation characteristics in patients have led to contradictory conclusions [7-11]. The mechanisms behind the oscillations are poorly understood mainly due to the variety of concomitant conditions. Moreover, most research relies on mathematical models, developed to generate common waveforms. Although mathematical models may be able to simulate recorded data of human eye movements, they are not necessarily biologically relevant. Therefore, an in depth study of the incidence and characteristics of nystagmus waveforms in an animal model is indispensable.

Recently, we introduced the zebrafish mutant *belladonna* (*bel*) as an animal model for INS [12-13]. In *bel* homozygous larvae, a variable fraction of optic nerve fibers are misrouted in the optic chiasm and project to the wrong brain hemisphere, a condition caused by mutations in *lhx2*, a Lim domain homeobox transcription factor [14-17]. Depending on the number of misprojecting fibers, *bel* larvae display INS-like ocular motor instabilities, such as a reversed optokinetic response (OKR) and spontaneous

eye oscillations with the same diagnostic waveforms reported in humans[12, 18-20]. Here, we investigate whether the incidence of different waveforms varies among *bel* individuals, thus reflecting different morphological conditions (i.e. optic nerve projection phenotypes). Moreover, we investigate how viewing conditions affect waveform characteristics.

Methods

All experiments were performed in accordance with the animal welfare guidelines of the Federal Veterinary Office of Switzerland. Experiments adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Fish maintenance and breeding

Fish were maintained and bred as previously described [21]. Embryos were raised at 28°C in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) and staged according to development in days post-fertilization (dpf). *bel* (*bel*$_{tv42}$) homozygous larvae were obtained from mating of identified heterozygous carriers. Larvae at 4 dpf were anesthetized with 200 mg/l 3-aminobenzoic acid ethyl esther methane sulfonate (MS-322, Sigma-Aldrich, Buchs, Switzerland) and sorted according to eye pigmentation phenotype [17].

Optokinetic response (OKR) and spontaneous oscillations

Larval eye movements were elicited as described previously [18, 22]. The presented stimulus was a computer-generated [23] black and white sine-wave grating pattern (contrast 85 % and maximum illumination 400 lux, spatial frequency 20 cycles/360

deg). Spontaneous oscillations were elicited by a stationary pattern; in complete darkness *bel* larvae do not show eye oscillations (ref). OKR was elicited by a rotating pattern (angular velocity 7.5 deg/s). Depending on the experiment, the pattern was presented binocularly or monocularly. Monocular stimulation was achieved by restriction of the visual field to one eye.

Eye movement recording and analysis

Binocular eye movements were recorded by an infrared-sensitive CCD camera (Guppy F-038B NIR, Allied Vision Technologies, Stadtroda, Germany). Frames were processed by a custom-developed tracking software based on LabView 2009 and NI Vision Development Module 2009 (National Instruments, Austin, TX, USA) with a frame rate of 25 frames/s. The software recognizes the eyes based on pixel intensity, extracts the angular position relative to the stimulation/recorded picture and calculates the velocity. Eye position and eye velocity traces were both used for the characterization of nystagmus waveforms. The relative frequency of a specific waveform was computed by dividing the total time of oscillations with this waveform by the total time of all oscillations during a recorded period (typically lasting xxx minutes). Since nystagmus was always conjugate, only the data from the right eye were used in the analysis. For analysis of orbital position relative to the larval body, movie frames were analyzed with the angle tool of ImageJ (MacBiophotonics, Hamilton, Ontario, Canada). Orbital position was defined by the angle between a transversal line below the eyes and a line that goes through the lens of the stimulated eye. Body pigmentation helped repositioning of the transversal line in the event of body movement.

Statistical analysis

Statistical analysis and graph generation were performed with SPSS Statistics 19 (IBM, Armonk, NY, USA). Influence of stimulus condition on the incidence of each waveform was analyzed using paired t-tests after transformation of percentage data using the formula , where x is the experimental data expressed as percentage. Since orbital position data were not normally distributed (Kolmogorov-Smirnov test), the relationship between orbital position and nystagmus waveform was analyzed in each larva using non parametric tests, i.e. Mann-Whitney U Test or Kruskal-Wallis Test. In the case of multiple comparison, the level of significance was adjusted for multiple testing.

Results

Categorization of nystagmus waveforms

Nystagmus in *bel* mutants matches the diagnostic waveforms of INS described by Dell'Osso and Daroff [12, 19]. For quantification of waveforms incidence, we grouped them in 3 main categories - pendular nystagmus, unidirectional jerk and bidirectional jerk - depending on the presence and direction of intercalated saccades (Fig. 1a). Pendular nystagmus is a sinusoidal oscillation without saccades or with only small breaking saccades. The absence of saccades is verified by examining the eye velocity trace. Unidirectional jerk consists of cycles of accelerating slow phases in one direction and breaking saccades in the opposite direction. Bidirectional jerk consists of cycles in alternating direction of slow phases and saccades. Saccades are seen as spikes in the eye velocity trace either always in the same direction

(unidirectional jerk) or in alternating direction (bidirectional jerk).

To quantify occurences and co-occurrences of waveforms in individual larvae, we selected time points of waveform change within the eye position trace. Figs. 1b-e show representative selected segments of eye position traces with changes in nystagmus waveforms. We frequently observed changes of waveforms without interruption of the ongoing oscillations (Figs 1b to 1c). If oscillations stopped for a certain period, the reappeared either with an accelerating slow drift of the eyes (Figs 1d) or after a saccade (Fig. 1e).

Co-occurrence of multiple waveform categories in single larvae

To investigate whether waveforms reflect different morphological phenotypes or are a single entity in the zebrafish *bel* mutant, we quantified the incidence of waveform categories during eye movement recordings in individual larvae. Waveforms of different categories occurred over time within the same larva, often without interruption of the oscillation (Fig. 2a). The three waveform categories co-occurred in 16 out of 22 larvae (Fig. 2b). Hence, classical waveform categories are not useful as predictors of specific morphological phenotypes. In 5 out of 22 larvae some cycles of unidirectional jerk with decelerating slow phases were observed. This waveform is characteristic of Fusion Maldevelopment Nystagmus (FMNS), but has been reported to occur for short periods in INS patients as well [20].

Influence of stimulus conditions on nystagmus waveforms

Next, we asked whether the incidence of waveform categories are influenced optokinetic stimulation. Traces of eye movements during monocular stimulation were

analyzed in each larva after a period of a unidirectional or directionally alternating optokinetic response (OKR). Unidirectional OKR, elicited by a rotating grating pattern changing direction every 30 s, is characterized by cycles of slow phases and resetting saccades. Bidirectional OKR, elicited by a rotating grating pattern changing direction every 2 s, is characterized by short slow phases in alternating direction without or with only few saccades. Unidirectional jerk occurred more often following a unidirectional OKR than following a bidirectional OKR ($p = 0.01$). In contrast, pendular nystagmus occurred more often following a bidirectional OKR than following a unidirectional OKR ($p = 0.013$). OKR properties did not have a significant effect on bidirectional jerk ($p > 0.05$) (Fig. 3a).

Traces of eye movements were analyzed in each larva during monocular or binocular stimulation. Unidirectional jerk occurred more often with a monocular field of view than with a binocular field of view ($p = 0.001$), while bidirectional jerk occurred more often with a binocular than with a monocular field of view ($p < 0.001$). Field of view did not have a significant effect on the incidence of pendular nystagmus ($p > 0.05$) (Fig. 3b).

Influence of orbital position on waveform initiation

Since waveform changes are observed under the same stimulus conditions, there must be larval intrinsic factors that change over time and influence the nystagmus waveform. In human patients waveforms are influenced by the eye position [8, 10-11, 19, 24-26]. To investigate whether orbital position has an effect on waveforms in *bel* larvae, we recorded eye movements during monocular stimulation and we measured orbital position of the stimulated eye when a new waveform was initiated. In 7 out of 13 measured larvae we found a significant tendency ($p < 0.05$) towards a defined orbital position when a period with a specific waveform started. In 3 of them (Class I),

pendular nystagmus was observed in eccentric, nasal position and jerk nystagmus started after a resetting saccade to a more central position (Figs. 4a and b). In 3 out of 7 larvae (Class II) unidirectional jerk started after a N-T saccade, short periods of bidirectional jerk - typically one cycle - started after a T-N saccade (Figs 4c and d). In 1 out of 7 larvae (Class III) bidirectional jerk was of higher amplitude than unidirectional jerk and therefore started at a more eccentric position (Figs 4e and f, see also Fig. 1b). However, in this case orbital position is likely not the direct cause of orbital change. In the remaining 6 out of 13 larvae we did not find a significant relationship between orbital position and waveform ($p > 0.05$): In one of those larvae we found a not significant tendency toward Class I (data not shown), in another larva a not significant tendency toward Class II (data not shown). In the remaining 4 larvae (Class IV) eyes oscillated around the central position and jerk waveforms were of similar amplitude (Figs. 4g and h).

Discussion

Research on the mechanisms underlying eye oscillations in INS has been based for a long time mainly on theoretical considerations and models due to the absence of suitable animal models. Recently, we introduced and characterized two new putative animal models displaying INS-like ocular motor abnormalities: Albino mouse strains and zebrafish *belladonna* mutant [12-13, 18, 27]. We demonstrated that in the *bel* mutant those abnormalities are caused by an aberrant decussation of retinofugal fibers at the optic chiasm leading to the projection of variable numbers of optic nerve fibers to the wrong brain hemisphere. We could show that additional eye morphological defects do affect visual performance in *bel* larvae but are not directly related to ocular motor abnormalities (ref). Thus, INS-like behavior in *bel* is explained by a normally

negative feedback loop turning into a positive loop, which increases retinal slip[13, 14]. In this study, we describe how nystagmus waveforms are influenced by intrinsic and extrinsic factors.

Incidence of nystagmus waveforms in bel

In agreement with the concept of a destabilizing positive feedback loop, we observed periods of spontaneous eye oscillations following a reversed OKR, a spontaneous saccade or starting with a spontaneous eye drift of exponential velocity (Fig. 1). The observation of spontaneous saccades as a triggering mechanism supports previous hypotheses considering saccades as a possible triggering mechanism [28-30], while being in disagreement with concepts that consider saccades in INS as purely of resetting nature [26],

Although only one mechanism - misrouting of optic nerve fibers - is at the origin of spontaneous eye oscillations in *bel* mutants, all characteristic INS waveforms are observed[12]. Our main aim was to investigate whether waveform categories reflect a different fraction of misprojecting optic nerve fibers. If this holds true, one would expect to see waveforms occurring predominantly in some fish but not in others. In contrast, we observed all main waveform categories co-occurring in the same individual in most cases (16 out of 22 larvae) whereby waveform changes were often observed without interruption of the oscillations (Fig. 2). These data suggest waveforms rather as a single entity in *bel* larvae.

Several factors may influence a sudden change in waveform or the relative incidence of different waveforms. Here, we investigated the role of viewing conditions and

observed a strong influence of both the properties of a triggering stimulus and the field of view (Fig. 3). Pendular nystagmus was more often observed after a bidirectional OKR than after a unidirectional OKR. In contrast, unidirectional jerk was more often observed after a unidirectional OKR than after a bidirectional OKR. Both bidirectional OKR and pendular nystagmus are characterized by a symmetric oscillation without or with only few saccades. In contrast, unidirectional OKR and unidirectional jerk both consist of cycles of slow phases and resetting saccades. Our data may indicate a possible biasing action of a preceding OKR on the spontaneous oscillation: The ocular motor system might tend to keep the oscillations characteristics when real motion of the visual word is stopped but retinal slip is maintained by the positive feedback loop.

We observed an effect of the visual field on the relative incidence of waveform categories. Unidirectional jerk occurred more frequently under monocular stimulation, whereas bidirectional jerk occurred more frequently under binocular stimulation. A possible explanation might lay in the preferential direction of nystagmus. Under monocular stimulation, a left-beating unidirectional nystagmus was more often observed if the left eye was stimulated and a right-beating unidirectional jerk was more often observed if the right eye was stimulated (data not shown). Under binocular stimulation, both eyes were stimulated and the nystagmus direction was biased to give rise to bidirectional jerk.

Despite the influence of environmental factors, waveform changes are often observed under the same stimulus conditions. Thus, changes in waveform must be triggered by intrinsic factors that change over time. A possible factor may be orbital position, shown to affect waveform changes in human INS patients (e.g. [8, 10-11, 19]). Here, we described a significant correlation between initiation of a specific waveform and orbital position in a subset of larvae (Fig.4). However, the effect of orbital

position in *bel* larvae is unclear and highly variable. In contrast to humans, zebrafish larvae are afoveate animals[31] so that the effect of gaze may be of less importance. Moreover, eye oscillations are of higher amplitude than in humans[12], often covering a big range of orbital positions within one cycle.

Significance for INS research

The mechanisms behind eye oscillations in INS are poorly understood. For a long time research was based on clinical observations and mathematical modeling. Theoretically, an infinite number of mathematical models can simulate real data, each starting from a different mechanism. Some models only simulate some waveforms but not others (e.g. [24-25]), thus different waveforms may reflect instabilities in different subsystems of the ocular motor system. Other models reproduce all waveforms starting from one mechanism (e.g. [28, 32]), suggesting that waveforms may be a single entity and occur together in one individual or reflect a different severity of one pathological mechanism. Experimental data can help shed light on which of those different modeled mechanisms really occur in nature.

The data presented here from the zebrafish *bel* mutant provide experimental support to models predicting INS waveforms as a single entity caused by reversal of a velocity feedback loop [25, 33]. Such a feedback reversal is also supported by studies in goldfish and amphibians, in which ocular motor instability were induced by surgically produced achiasmia[34] or by rotation of the eye balls by 180 deg[35].

Waveforms do not reflect the severity of the underlying pathology, but are rather influenced by the viewing conditions, e.g. properties of the triggering stimulus, field of view, and, partially, orbital position. Waveforms changes under unaltered stimulus

properties might depend on intrinsic factors such as eye position and eye velocity, regulated by the naturally fluctuating activity of a neural integrator network[25] and/or by the activity of the saccadic system[28]. Psychological factors, including stress, fatigue, and level of attention have been suggested as further influencing factors in humans [8, 36]. Although in the zebrafish *bel* mutant INS is caused by optic nerve fibers misprojections, a reversed feedback loop could also be caused by other aberrant projections, e.g. of afferents from extraocular muscles [25].

Here, we only studied the incidence of waveforms in the presence of a reversed feedback loop. We did not investigate the possibility that different mechanisms can lead to the same oscillations and we cannot exclude some differences in relative incidence of waveform categories among groups with different background conditions, as described by Kumar et al. [11].

Conclusions

We have described an experimental model of INS in which one pathological mechanism leads to all classical waveform categories within one individual. Therefore, waveforms do not reflect the severity of the disease, suggesting that classical waveform categories are unlikely to provide much diagnostic benefit. Our findings of a strong influence of viewing conditions on nystagmus waveforms suggest that it may be difficult to compare different studies in which conditions are not identical. Our observation are not only of benefit for clinical research but may also help elucidating how changes in ocular motor control systems influence eye movement.

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Figure legends

Figure 1: Nystagmus waveforms

A stationary grating pattern was presented to elicit spontaneous eye oscillations. Movements of the right eye are shown. A higher value on the y-axis indicates a more temporal position.

A) Representative eye position traces ((Se (deg)) are shown together with corresponding eye velocity traces (Ve (deg/s)). Arrows in the eye velocity trace indicate saccades.

B-E) Representative oscillations with waveform change are shown on eye position traces and their corresponding eye velocity traces. Arrows indicate the time point of waveform change. In B) a change from unidirectional jerk to bidirectional jerk is shown: After a saccade, the decelerating eye increases its velocity again before turning to the opposite direction. In C) a change from unidirectional jerk to pendular nystagmus is shown: A saccade is replaced by a slow eye movement. In D) and E) a starting unidirectional jerk after a period without oscillations is shown. A period with nystagmus started either with an accelerating eye drift (D) or after a spontaneous saccade (E).

Figure 2: Co-occurring waveforms

A stationary grating pattern was presented to the full field of view of both eyes (binocular stimulation) during 5 minutes. Movements of the right eye were used for analysis.

A) Representative segment of an eye position trace (Se (deg)) is shown together with the corresponding eye velocity trace (Ve (deg/s)). The 3 main waveform categories occurred without interruption of the oscillations in this larva. Pendular nystagmus

(violet horizontal bar) is followed by unidirectional jerk (green horizontal bar) and by bidirectional jerk (blue horizontal bar). A higher eye position on the y-axis indicates a more temporal position. # indicates a body movement artifact.

B) Stacked bar graph showing the incidence of spontaneous oscillations (SOs) waveforms in individual larvae. Asterisks indicate larvae that displayed all main waveforms within one recording (16 out of 22 larvae). 5 larvae displayed periods of unidirectional jerk with decelerating slow phases.

Figure 3: Influence of stimulus conditions on nystagmus waveforms

A) A stationary grating pattern was presented to one eye (monocular stimulation) during 1 minute. Movements of the right eye were used for analysis. Spontaneous oscillations (SOs) waveforms were determined within a period starting right after the OKR and ending when the oscillation discontinued. Data were considered if nystagmus lasted for at least 15 seconds without interruption. For each waveform category, the difference in incidence (Δ % of period with SOs) after a bidirectional OKR and after a unidirectional OKR was calculated and plotted (mean ± SEM; n = 16). The horizontal line indicates no difference in incidence between the two conditions. A negative value indicates a higher incidence after unidirectional OKR and a positive value indicates a higher incidence after bidirectional OKR. * $p < 0.05$; ns $p > 0.05$.

B) A stationary grating pattern was presented during 5 minutes. Movements of the right eye were used for analysis. For each waveform category, the difference in incidence (Δ % of period with SOs) under binocular and monocular visual field stimulation was calculated and plotted (mean ± SEM; n = 20). The horizontal line indicates no difference in incidence between the two conditions. A negative value indicates a higher incidence with a monocular field of view, a positive value indicates

a higher incidence with a binocular field of view. ** $p < 0.01$; *** $p < 0.001$; ns $p > 0.05$.

Figure 4: Influence of orbital position on waveform initiation

A stationary grating pattern was presented to one eye (monocular stimulation) during 10 minutes. Orbital position of the stimulated eye was quantified at time points of waveform change as described in the methods section. Statistical analysis was performed in each larva separately. Data were considered for statistical analysis if of at least 2 waveform categories at least 5 periods occurred in one recording. Waveforms occurring for less than 5 periods were not considered.

Phenotypes were clustered in 4 classes. For each phenotype class, data from a representative larva are shown. On the left, Box-and-whisker plots of the orbital position at begin of periods with each waveform category are shown. Circles represent outliers. Dashed horizontal line represents the central orbital position. A lower value indicates a more nasal position. *n* = number of periods with a specific waveform category within one recording. ** $p < 0.01$; *** $p < 0.001$. On the right, representative segments from the eye position trace (Se (deg)) and the corresponding eye velocity trace (Ve (deg/s) are shown. A higher eye position on the y-axis indicates a more temporal position. Arrows indicate the time point of waveform change.

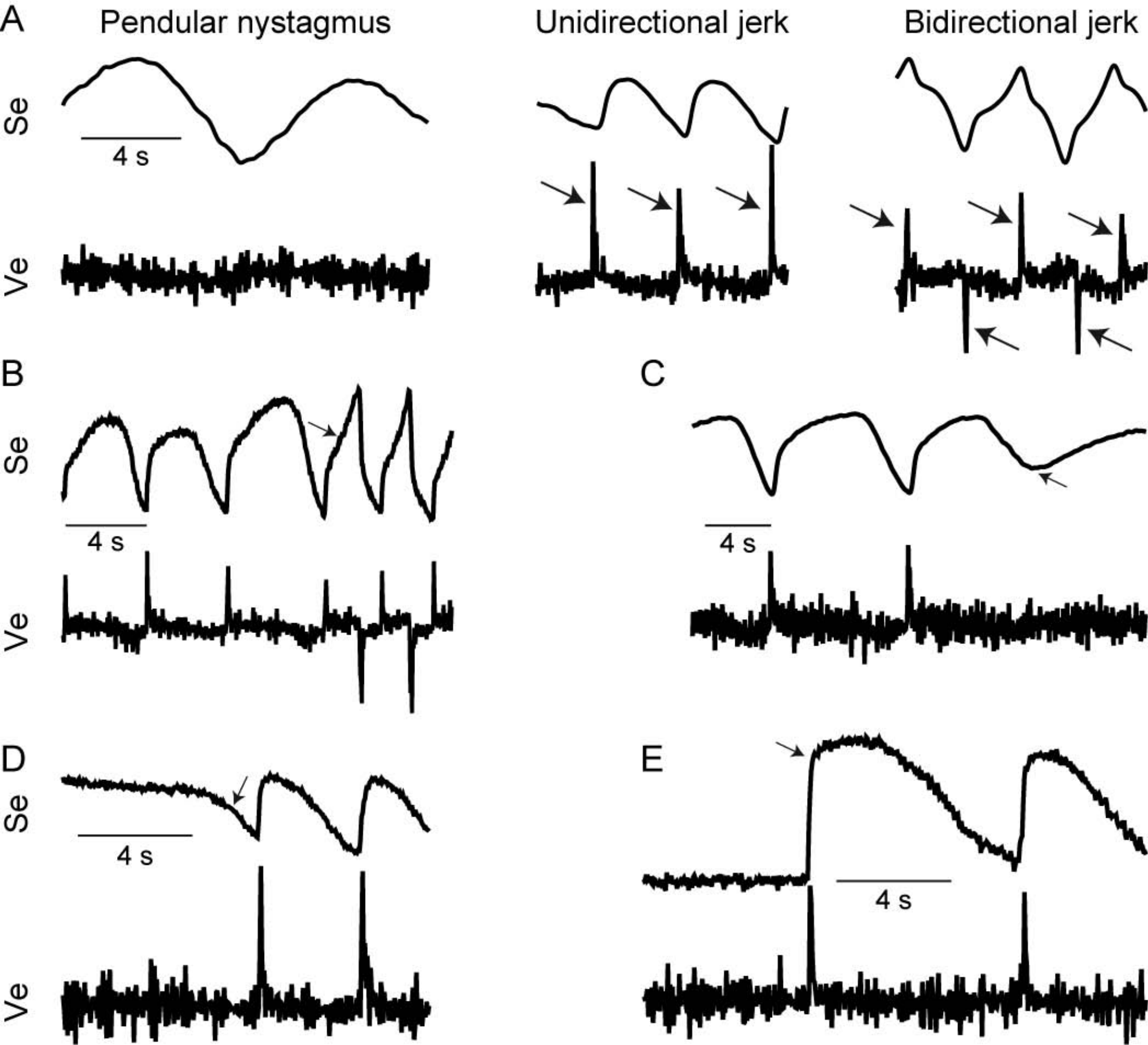
A-B) Class I phenotype. Pendular nystagmus is observed on eccentric orbital position, unidirectional jerk starts after a resetting saccade.

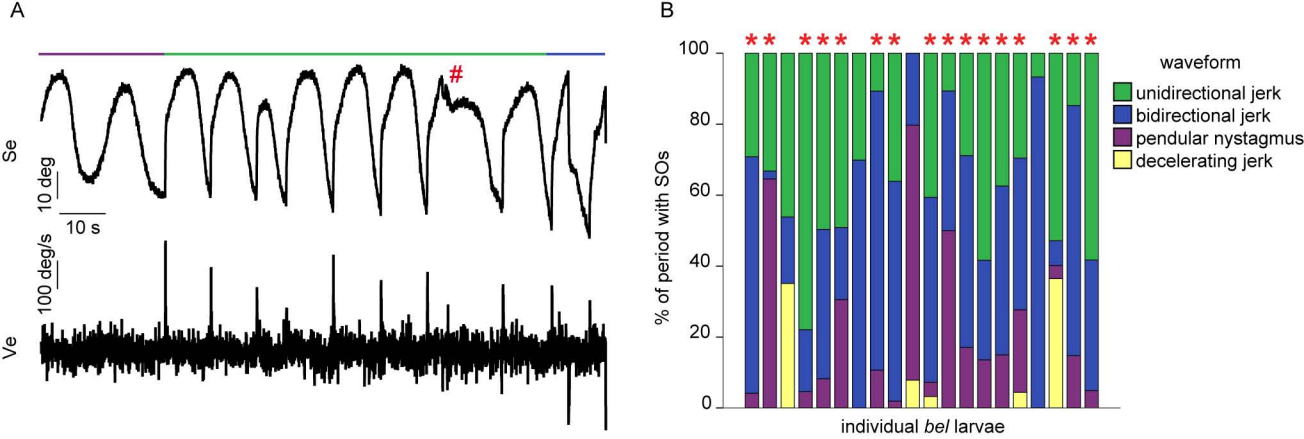
C-D) Class II phenotype. Unidirectional jerk starts after a N-T saccade, bidirectional jerk after a T-N saccade.

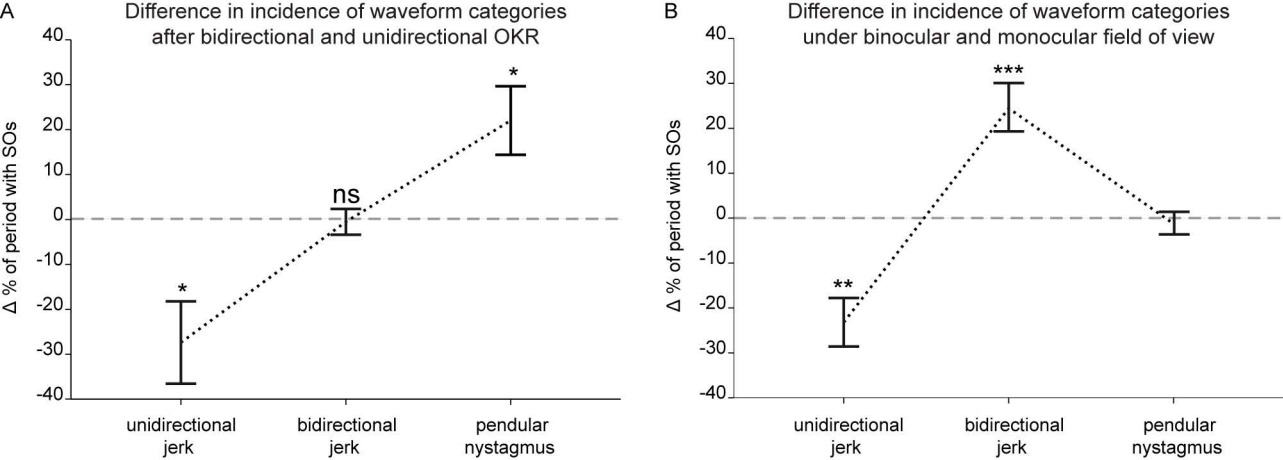
E-F) Class III phenotype. Bidirectional jerk cycles are characterized by a higher

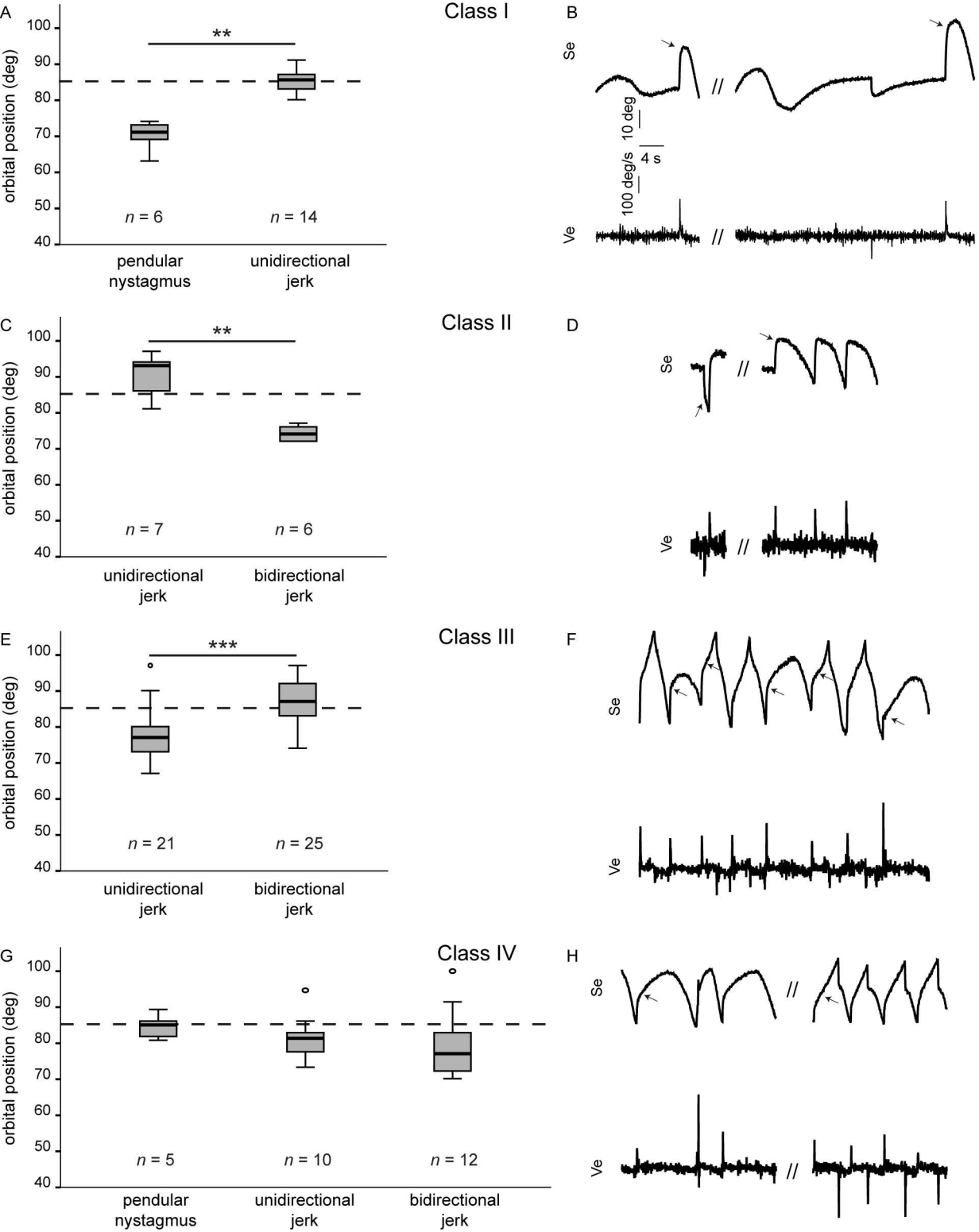
amplitude.

G-H) Class IV phenotype. Orbital position does not influence waveform changes.


A
Pendular nystagmus
Unidirectional jerk
Bidirectional jerk
Se
Ve
4 s
B
C
D
E


A
Se
10 deg
10 s
100 deg/s
Ve
#
B
% of period with SOs
100
80
60
40
20
0
individual *bel* larvae
waveform
unidirectional jerk
bidirectional jerk
pendular nystagmus
decelerating jerk


A
Difference in incidence of waveform categories
after bidirectional and unidirectional OKR
Δ % of period with SOs
40
30
20
10
0
-10
-20
-30
-40
*
ns
*
unidirectional
jerk
bidirectional
jerk
pendular
nystagmus
B
Difference in incidence of waveform categories
under binocular and monocular field of view
Δ % of period with SOs
40
30
20
10
0
-10
-20
-30
-40
**
***
unidirectional
jerk
bidirectional
jerk
pendular
nystagmus


A
Class I
B
**
orbital position (deg)
100
90
80
70
60
50
40
n = 6
n = 14
pendular nystagmus
unidirectional jerk
Se
Ve
10 deg
4 s
100 deg/s
C
Class II
D
**
n = 7
n = 6
unidirectional jerk
bidirectional jerk
E
Class III
F
***
n = 21
n = 25
G
Class IV
H
n = 5
n = 10
n = 12
pendular nystagmus
unidirectional jerk
bidirectional jerk